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Characterization of the Hydrolytic Activity of Avocado Cellulase[1]

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The cellulase produced by ripening avocado fruits (*Persea americana* Mill cv. Fuerte) was isolated and purified using chromatofocusing (pH 7–4) and gel filtration on a Bio-Gel P-100 column. Characteristics of the cellulase were assessed by using, as substrates, a range of polysaccharides containing various sugar residues and varying types of linkages between the residues. Only those substrates containing (1→4)-β-glucosyl linkages were hydrolyzed by the purified enzyme. Two polysaccharides that were extensively hydrolyzed by the cellulase were carboxymethylcellulose and (1→3),(1→4)-β-D-glucans such as from *Avena* endosperm cell walls. Characterization of the activity in the degradation of the mixed linked glucan of *Avena* and cellodextrins indicated that the enzyme has a limit recognition-hydrolytic site of four (1→4)-β linked glucose residues. It was also found that the enzyme could cleave only (1→4)-β-linkages that were adjacent to other (1→4)-β-D-glucosyl linkages. Activity of the cellulase against isolated avocado fruit cell walls indicated that the purified enzyme was incapable of appreciably solubilizing the cellulosic components of these walls.

Key words: Carboxymethylcellulose — Cellulase (avocado) — Glucan — *Persea americana*.

There are several reports documenting the prodigious cellulase activity in ripening fruits (Awad and Young 1979, Barnes and Patchett 1976, Roe and Bruenmer 1981, Sobotka and Stelzig 1974, Yamaki and Kakiuchi 1979). It has been assumed that this hydrolytic activity plays a role in cell wall modification leading to fruit softening. Generally this assumption is based on the observation that initial enzyme activity is low or absent in unripe fruit and increases rapidly during the ripening process. The exact role of cellulase activity in ripening fruits is not clear. In many cases the specificity of cellulases has not been characterized in sufficient detail to determine if multiple forms of the enzyme are present during ripening. Some investigators have suggested that cellulase plays a minor role during fruit ripening based upon the observation that little or no cellulosic glucan is lost during this process (Ahmed and Labavitch 1980, Gross and Wallner 1979). Less direct evidence has been presented by Platt-Aloia et al. (1980) utilizing electron microscopy. They observed a loss of organization, density of wall striations, and a loss of fibrillar components during the late stages in the softening of avocado fruits. Because

Abbreviations: CMC, carboxymethylcellulose; RS, reducing sugars; TS, total sugar; TFA, trifluoracetic acid; D.P., degree of polymerization; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry.

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of the large increase in cellulase activity during this process they suggested that this enzyme is responsible for fibrillar disruption and subsequent loss of wall integrity.

In nearly all reports of cellulase during fruit ripening the activity identified has been of the carboxymethylcellulase, Cx, (EC 3.2.1.4) type. Although such enzymes have been shown to depolymerize CMC, little or no activity has been demonstrated against insoluble or crystalline forms of cellulose (Reese 1977). An exception is the report by Sobotka and Stelzig (1974) of a cellulase complex in ripening tomato. They identified four enzymes that could act in conjunction to degrade native cellulose; a β-glucosidase, an exo-β-(1$\rightarrow$4)-glucanase, and two endocellulases. To date no other cellulase complex has been described in ripening fruits that can degrade native cellulose.

Evidence for a role of cellulase in ripening avocado fruits is based on ultrastructural changes already mentioned and the rapid increase in activity during the climacteric (Awad and Young 1979). According to Awad and Young (1979) the cellulase increase preceded the increase in polygalacturonase activity by about three days. The strong correlation between cellulase activity and softening suggests that in avocado the initial phase of softening may be due to cellulase.

Although there are strong correlations between fruit softening and increased cellulase activity for avocado fruits, there is no direct evidence of hydrolytic activity against native cellulose. Since avocado cellulase represents a unique event in plant development the work described here was undertaken to characterize the hydrolytic activity of this enzyme. In addition, this study is an attempt to determine the substrate specificity of the enzyme and to assess its putative role in degradation of avocado cell walls.

Materials and Methods

Plant material—Avocado (*Persea americana* Mill. cv. Fuerte) fruits were purchased from a local retail outlet. Individual fruits were allowed to ripen for 4–6 d until the texture of the fruit was soft. Initial experiments indicated that this stage corresponded roughly with maximum cellulase activity. Cellulase was extracted from soft avocado fruits using the procedure of Awad and Lewis (1980) with minor variations. Homogenates were incubated in the extraction buffer for 20 min at 0–4°C, then centrifuged at 8,000$\times g$ for 20 min. The supernatant was carefully removed with minimal disruption of the lipid layer at the top of the centrifuge tube. This lipid layer was discarded.

Pelleted material was resuspended in extraction buffer and filtered through Miracloth. After washing with 50 mM NaCl solution the residue was extensively washed with cold acetone (−20°C). The wall material was again washed with 50 mM NaCl solution to remove acetone, resuspended in distilled water, and autoclaved for 20 min. The resulting inactivated cell wall residue was freeze-dried and served as native cell wall substrate. Cell walls were also isolated from green fruits at stages prior to the expression of cellulase activity.

Purification of cellulase—Purification of cellulase involved fractionation of the extract (supernatant) with $(NH_4)_2SO_4$. The initial fraction (20% saturation) contained a small amount of cellulase activity and was discarded. The second fraction (80% saturation) contained 80–90% of the total cellulase activity, based on the hydrolysis of CMC 7M. The precipitate was dialyzed against 20 mM acetate buffer (pH 5.0 20 mM NaCl) for 24 h with two changes of buffer. After dialysis the samples were centrifuged at 10,000$\times g$ for 20 min to remove insoluble material. The supernatant was concentrated to 2–5 ml using an Amicon Stirred Ultrafiltration Cell (Model 52, Diaflo membrane PM-10). The concentrated cellulase extract was dialyzed against 0.025 M imidazole-HCl buffer (pH 7.4) for 4–6 h then applied to a chromatofocusing column (1.0$\times$30 cm) PBE 94 (Pharmacia), equilibrated with the same buffer. Protein was eluted with 200 ml of Polybuffer 74 adjusted to pH 3.8 with 1 M HCl. The Polybuffer was pumped through

the column at a flow rate of 8 ml/h. Fractions (2 ml) were collected and assayed for protein (A_{280}). Cellulase activity in selected fractions was assayed by rate of change in viscosity of a 15% solution of CMC 7M in 20 mM acetate buffer (pH 5.0). For each assay 0.5 ml of CMC solution was incubated with 0.05 ml of a column fraction. Changes in viscosity were monitored by changes in drainage time of the reaction mixture from a standard 0.2 ml pipette. All assay tubes were maintained at 30°C and viscosity measurements were taken every 10 min for 1 h.

Fractions containing the highest activity were pooled and dialyzed against 10 mM citrate-phosphate buffer (pH 5.6, 200 mM NaCl) for 10–12 h. The dialyzed fractions were concentrated to a volume of 5–6 ml and applied to a Bio-Gel P-100 (100–200 mesh) column (3.0 × 65 cm) equilibrated with the same buffer. Head pressure was maintained to give a constant flow rate of 15 ml/h. Two ml fractions were collected and assayed for protein (A_{280}) and cellulase activity. The cellulase activity of individual fractions was determined by incubating 0.1 ml from a column fraction with 0.4 ml of a CMC solution (2 mg/ml) in the acetate buffer. After 12–16 h at 30°C the increase in reducing sugar equivalents was determined.

Hydrolytic activity—The hydrolytic activity of the purified cellulase was evaluated with a range of substrates. Activity was determined by an increase in the reducing sugar equivalents released from a substrate after 24 h incubation at 30°C. Routinely, substrates were prepared in 20 mM acetate buffer (pH 5.0) at a concentration of 1 mg/ml. One ml of each substrate was incubated with 2.5 μg of cellulase.

The hydrolytic action pattern of cellulase upon CMC 7M was monitored by measuring the changes in viscosity as a function of incubation time. Viscosity changes were measured using a Cannon-Manning Semi-Micro Viscometer (# 300) to which 5 ml of 15% CMC 7M was added. The viscometer was maintained at 30°C by immersion in a circulating waterbath. When temperature equilibrium was reached, 50 μl of cellulase (220 μg/ml) was added to the viscometer with thorough mixing. At given time intervals the viscosity was determined and aliquots (100 μl) were removed and assayed for reducing sugar equivalents.

To assist in the determination of the recognition-hydrolytic limits of the avocado cellulase the capacity of the enzyme to act on (1→3),(1→4)-β-D-glucan (β-D-glucan) from *Avena* endosperm was investigated in greater detail. A sample of *Avena* β-D-glucan (25 mg) was dissolved in 20 mM acetate buffer, containing 0.02% NaN_3 to suppress microbial growth, and incubated with avocado cellulase (50 μg) for 30 h. Samples were removed after incubation for fractionation on Bio-Gel A1.5M (1.5 × 60 cm), Fractogel TSK HW 50-F (1.0 × 125 cm), and Bio-Gel P-2 (1.5 × 150 cm) columns. Fractions from the P-2 profile were analyzed by paper chromatography and by methylation analysis.

The rate of hydrolysis of cellodextrins was determined as follows. Cellodextrins ranging from cellobiose to cellopentaose were prepared in acetate buffer at a concentration of 100 μg/ml. Cellulase was added to each cellodextrin substrate at a final concentration of 10 μg/ml. Aliquots (200 μl) were removed from the total reaction mixture at 0, 0.5, 1.0, 2.0 and 4.0 h and assayed for reducing sugar equivalents.

The hydrolytic activity of avocado cellulase against isolated cell wall preparations was determined using cell walls from two stages ("green" and "ripe") of avocado fruits isolated as described under enzyme extraction. Samples of each cell wall preparation were resuspended in 20 mM acetate buffer (pH 5.0). Aliquots of approximately 5 ml of each cell wall substrate was incubated with 25 μg of enzyme for 72 h. After incubation each sample was filtered over a glass filter with the aid of suction and the filtrate heated in boiling water 10 min. Total sugar and neutral sugar analysis was performed on each filtrate. A commercial cellulase preparation, (Worthington, CELF 34 units/mg) was included as a treatment to assess the potential of each cell wall preparation to release glucose and other neutral sugars.

Carbohydrates used in enzyme characterization—The following carbohydrates were tested as

enzyme substrates: larch xylan, starch, dextran, arabinogalactan, and lichenan from Sigma Chemical Co.; araban and nigeran from Koch-Light Ltd.; polygalacturonan from Sunkist; laminarin from United States Biochemical Co.; carboxymethylcellulose (CMC type 7M and 4M) from Hercules; hydroxyethylcellulose (type H and M) from Polysciences Inc.; Avicel PH-101 from FMC Corporation; and *Avena* (1→3),(1→4)-β-D-glucan from Quaker Oats Co. Laminarin from *Esenia bicyclis* was isolated by R. Yamamoto as described by Yamamoto and Nevins (1983). Xyloglucan was prepared from soybean hypocotyl by Y. Kato using the procedure of Kato and Matsuda (1976).

General methods—Total sugar assays were performed using the phenol-H_2SO_4 method (Dubois et al. 1956). Reducing sugar equivalents were determined using the method of Nelson (1944) as modified by Somogyi (1952). Protein was determined in column fractions by monitoring A_{280} or in pooled fractions by the Bio-Rad Protein Assay (Bio-Rad) using BSA (Sigma) as a standard. Paper chromatographic analysis of oligosaccharide hydrolytic products was performed on Whatman No. 1 paper using the multiple ascent method with 1-butanol : pyridine : water (6 : 4 : 3) (v/v/v). Sugars on the chromatogram were detected with alkaline silver nitrate (Roybt and French 1963).

Neutral sugar composition analysis was carried out on 250 μg (as glucose equivalents) of released carbohydrate. Samples were hydrolyzed with 2 M TFA for 1 h at 121°C. Sugars were converted to corresponding alditol acetates and analyzed by GLC on a glass column (0.2 × 190 cm) packed with 3% SP-2340 (Supelco) at 210°C with a helium flow rate of 35 ml/min.

Methylation analysis of selected carbohydrate samples was performed according to the procedure of Harris et al. (1984). The partially methylated alditol acetates were analyzed by GLC on a 30 meter DB-1 fused silica capillary column at a temperature range from 150–230°C (4°C/min) with a split ratio of 50 : 1. Some samples were initially reduced with $NaBD_4$ then subjected to methylation analysis using GLC-MS. The partially methylated alditol acetates were chromatographed on a fused silica crosslinked methyl silicone (Hewitt-Packard) 0.2 mm × 25 m capillary column using a Hewitt-Packard 5890 gas chromatograph interfaced with a Hewitt-Packard 5970 Series Mass Selective Detector.

Results and Discussion

Enzyme purification—Purification of the cellulase was accomplished by essentially a three step procedure involving ammonium sulfate fractionation, chromatofocusing (pH range 7–4), and gel filtration on Bio-Gel P-100. The chromatofocusing step effectively separated the cellulase activity from a major portion of the total protein (Fig. 1). In most cellulase preparations the chromatofocusing column resulted in two peaks of activity when assayed by viscosity changes of 15% CMC solution. Although these two peaks were initially separated and subjected to further purification, general properties and activities appeared to be similar. Chromatography on Bio-Gel P-100 resulted in a single peak of activity at an apparent molecular weight of approximately 45 kDa (Fig. 2). Both cellulase peaks from the chromatofocusing column eluted at the same position. Characterization of the hydrolytic capacity of the individual peaks did not reveal any differences. Therefore reference will be made to cellulase activity without distinguishing between the two. It is possible that the cellulase exists in two forms with slightly different isoelectric points but with an expression of the same hydrolytic pattern. The purification procedure resulted in approximately 450 fold increase in specific activity.

Substrate specificity—Initially several different polysaccharides were used as substrates to assess the purity of the cellulase preparation in terms of contaminating hydrolytic activity. The substrates tested included arabinogalactan containing (1→3)-β and (1→6)-β-galactopyranosides with α-arabinofuranosidic branches; galactan containing (1→4)-β-galactopyranosidic linkages;

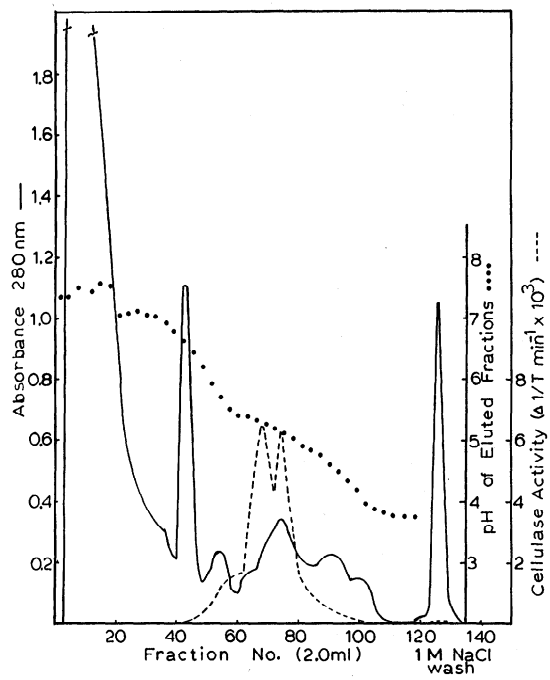

Fig. 1

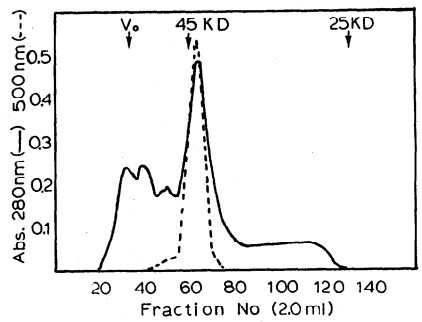

Fig. 2

Fig. 1 Chromatofocusing profile of proteins resolved from the 80% $(NH_4)_2SO_4$ fraction of crude avocado extract. The large peak eluting at the void volume of the column was due, in part, to non-protein UV absorbing material.

Fig. 2 Bio-Gel P-100 profile of pooled cellulase fractions from the chromatofocusing column.

larch xylan containing (1→4)-β-xylopyranosidic linkages; polygalacturonic acid containing (1→4)-α-D-galactouronopyranosidic linkages; araban containing (1→5)-α- and (1→3)-α-arabinofuranosidic linkages; and the α-D-glucans starch, nigeran, and dextran. No hydrolytic activity against these substrates was detected in the purified enzyme preparation.

Substrates of particular interest were the β-D-glucans especially those with (1→4)-β-linkages. Table 1 gives a summary of the avocado cellulase activity against polysaccharides representative of the major types of β-D-glucan. The substrates hydrolyzed to the greatest extent were CMC and the (1→3),(1→4)-β-D-glucans.

The degree of hydrolysis of the CMC molecule appeared to be directly affected by the type and degree of substitution on the cellulose molecule. Carboxymethylcellulose 7M has an average of 7 carboxymethyl groups per 10 glucose units for an average substitution of 0.7 (range 0.65–0.85) carboxymethyl groups per glucose. Carboxymethylcellulose 4M has an average substitution of 0.4 (range 0.38–0.48) carboxymethyl groups per glucose molecule. The hydrolytic activity against CMC 4M is over twice as high as with CMC 7M (Table 1), suggesting a preference of the enzyme for either longer unsubstituted or occasionally substituted regions along the glucose polymer.

The modified cellulose, hydroxyethylcellulose, was not hydrolyzed to the same extent as CMC. Both medium (M) and high (H) viscosity forms were readily soluble in buffer and were degraded to approximately the same extent. The low reactivity may be a result of the form of the modifying groups substituted on the glucose residues. In many cases the hydroxyethylether substituents are not single groups but rather dihydroxyethylether side chains. These extended

Table 1 Analysis of substrate specificity of avocado cellulase using various β-D-glucans

Substrate	Reducing sugar equivalents μg RS/mg TS/μg protein
β-(1→3) linkages	
Laminarin, *Laminaria digitata*	0.0
Laminarin, *Eisenia bicyclis*, also (1→6)	0.0
Pachyman	0.0
β-(1→4) linkages	
Carboxymethylcellulose 7 M	33.4
Carboxymethylcellulose 4 M	72.8
Hydroxyethylcellulose M	4.6
Hydroxyethylcellulose H	4.5
Avicel (swollen cellulose)	0.0
Xyloglucan, soybean hypocotyl	2.8
Intramolecular β-(1→3), β-(1→4) linkages	
β-D-Glucan *Avena* endosperm	68.8
Lichenan, *Cetraria islandica*	28.4

side groups are bulkier than the carboxymethyl groups and could interfere with the binding of the enzyme to the glucan polymer. This would seem to be particularly true if the enzyme has an endo-type of action pattern requiring more than 3–4 unsubstituted glucose residues for effective binding and hydrolysis.

Avicel, which was prepared according to the procedure of Wood (1971) to give a swollen form of native cellulose, was not degraded by the avocado cellulase. Since activity was judged by an increase in reducing sugar equivalents, it is possible that the enzyme could cleave random internal glucosidic linkages. If this activity was relatively restricted, the production of soluble oligosaccharides may not occur and no release of reducing sugars into the supernatant would be expected. The only other type of substrate that was actively hydrolyzed were the (1→3),(1→4)-β-D-glucans. These substrates were hydrolyzed in a manner similar to the CMC molecules.

Hydrolytic action pattern—The action pattern of avocado cellulase against CMC 7M is characteristic of an endohydrolase as shown in Fig. 3. There is an initial rapid decrease in viscosity with a linear increase in RS equivalents. The increase in RS equivalents produced is relatively low as compared to the decrease in viscosity. This combination indicates that the enzyme initially hydrolyzes internal glucosidic bonds producing large polymeric fragments. The increase in RS equivalents was linear up to 12 h after which there was little increase over the next 12 h. The leveling off in production of RS equivalents suggests that the enzyme has exhausted all the possible hydrolytic sites within the CMC polymer.

In order to develop a better understanding of the hydrolytic activity of avocado cellulase, the limit products were examined from exhaustive hydrolysis of *Avena* (1→3),(1→4)-β-D-glucan. The structure of the *Avena* β-D-glucan molecule has been investigated by Peat et al. (1957) providing information that would assist in the interpretation of the origin of released fragments. A comparison of hydrolytic products from CMC 7M and *Avena* β-D-glucan is shown in Fig. 4. The β-D-glucan was hydrolyzed to a greater extent releasing fragments that appear to be relatively small in terms of molecular weight. A more precise determination of the molecular weight distribution of *Avena* β-D-glucan hydrolysis products was obtained from the fractionation of

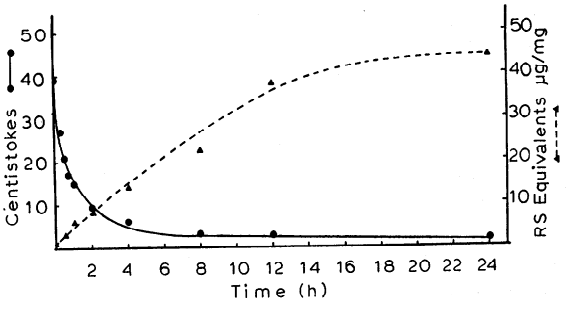

Fig. 3

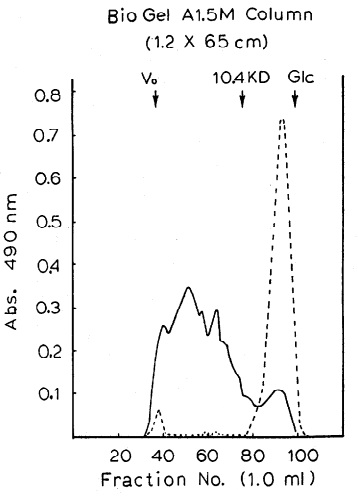

Fig. 4

Fig. 3 Hydrolytic activity of purified avocado cellulase against CMC 7M (15 mg/ml). (——) viscosity change, (----) increase in reducing sugar equivalents.

Fig. 4 Bio-Gel A1.5M profile of CMC 7M (——) and *Avena* glucan (----) after 24 h incubation with avocado cellulase. Both substrates eluted in the void volume before hydrolysis by the cellulase.

hydrolytic products on a Fractogel TSK HW 50F column. Generally the products released appeared to be distributed between fragments corresponding to a D.P. of 3 and 30.

To assess the limit recognition-hydrolytic site of this enzyme, *Avena* β-D-glucan was incubated with avocado cellulase at an enzyme concentration of 2 μg/mg of glucan. The total time of incubation was extended to 30 h at 30°C. The products were fractionated on a Bio-Gel P-2 column (Fig. 5). A portion (50–200 μg by glucose equivalents) of fractions A, B, C and D, were reduced with $NaBD_4$ and subjected to methylation analysis. By reducing the oligosaccharides before methylation it was possible to determine the linkage position on the reducing end glucosyl residue. Reduction of the reducing residue prior to methylation should result in the production of either 3-O-acetyl-1,2,4,5,6-penta-O-methyl-D-glucitol or 4-O-acetyl-1,2,3,5,6-penta-O-methyl-D-glucitol. The type of product will depend upon whether the reducing glucose residue was (1→3)-β- or (1→4)-β-linked. The only residue detected was 4-O-acetyl-1,2,3,5,6-penta-O-methyl-D-glucitol for the oligosaccharides in A, B, C and D. This was based on a comparison of the retention time on a capillary column of GLC with the retention time of methylated glucitol residues produced from reduced cellobiose as a standard. Identity of the methylated sugars was confirmed by GLC-MS.

The evidence for only (1→4)-β- linked glucosyl residues at the reducing terminal in these fractions suggests that the cellulase hydrolyzes an internal (1→4)-β- linkage as opposed to a linkage adjacent to a (1→3)-β- linkage. It is also evident from the Bio-Gel P-2 profile that the enzyme has a limit recognition-hydrolytic site of four (1→4)-β- linked glucosyl residues. This is based on the structural information that is known for *Avena* β-D-glucan. This β-D-glucan molecule is composed of randomly arranged cellotriose and cellotetraose oligosaccharides separated by single (1→3)-β-glucosyl linkages (Peat et al. 1957). If this enzyme has an action pattern similar to that of the *Streptomyces* cellulase, which produces fragments of D.P. 3 and 4 from *Avena* β-D-glucan, larger molecular weight fragments should not have been produced. The *Streptomyces* cellulase requires a minimum of a tetrasaccharide but the linkage of the reducing

end residue can be either (1→4)-β- or (1→3)-β-. Hydrolysis by *Streptomyces* cellulase occurs at a (1→4)-β-linkage that is adjacent to another (1→4)-β- glucosyl linkage on the non-reducing terminal side (Anderson and Stone 1975, Parrish et al. 1960, see Fig. 5). The hydrolytic pattern of avocado cellulase suggests that this enzyme hydrolyzes consecutively linked (1→4)-β-glucosyl residues of D.P. 4 or greater.

When a higher molecular weight fraction such as E, was treated with the β-D-endoglucanase from *Bacillus subtilis* (EC 3.2.1.73), one major product was released which elutes at the D.P. 3 region on a Bio-Gel P-2 column. Methylation analysis of this peak indicates that it is composed of approximately equal amounts of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol. This would indicate that the fragment released by the *B. subtilis* endoglucanase was 3-O-β-cellobiosyl-D-glucose. The second major fraction appears to be composed of a mixture of cellobiose, laminaribiose and possibly a small amount of trisaccharide containing 1 or 2 1→3 linkages.

Treatment of fraction D (Fig. 5) with *B. subtilis* endoglucanase resulted in two major peaks. One corresponding to a D.P. 3 and a larger peak eluting in the D.P. 2 region. Paper chromatography of the D.P. 2 fraction indicated two major oligosaccharides, one which co-migrated with cellobiose and one with laminaribiose. Methylation analysis indicated that (1→3)-β- and (1→4)-β- combinations in higher oligosaccharides were also present. Methylation analysis of fraction D.P. 3 indicated it contained mostly 3-O-β-cellobiosyl-D-glucose. Analyses of all the products digested by the *Bacillus subtilis* enzyme would reinforce the conclusion that the cellulase activity is restricted to the cellotetraose regions of the *Avena* β-D-glucan molecule.

From the results of activity against cellodextrins (Table 2) it would appear that the avocado cellulase hydrolyzes oligosaccharides of D.P. ≥ 5 more readily than oligosaccharides of D.P. ≤ 4.

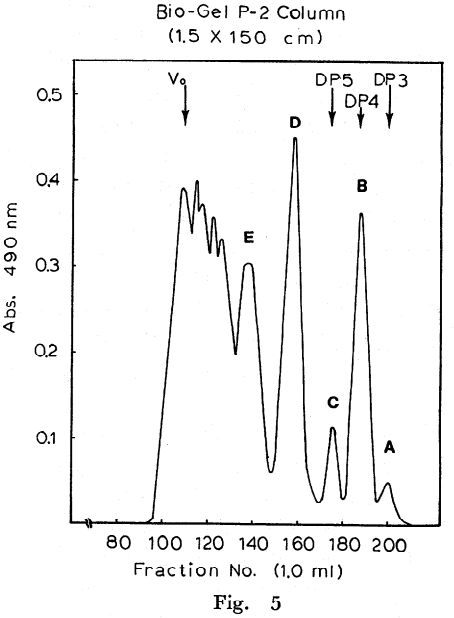

Fig. 5

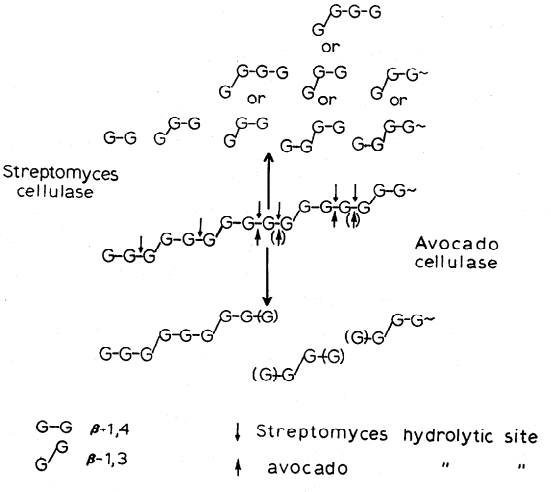

Fig. 6

Fig. 5 Bio-Gel P-2 profile of oligosaccharides released from *Avena* glucan by avocado cellulase.

Fig. 6 Hydrolytic pattern of *Streptomyces* cellulase and a proposed hydrolytic pattern of avocado cellulase activity on mixed linked (1→3),(1→4)-β-D-glucans.

Table 2 Analysis of substrate specificity of avocado cellulase against β-(1→4) oligosaccharides (0.1 mg/ml)

Oligosaccharide D.P.	Reducing sugar equivalents generated μg RS/h/μg protein
2	0.0
3	0.67
4	1.08
5	2.12

There was also some hydrolysis of oligosaccharides of D.P. 3. This would seem to contradict the results obtained from the exhaustive hydrolysis of *Avena* β-D-glucan. However, it is possible that the oligosaccharide (D.P. 3) can bind to the active site and is hydrolyzed whereas a region containing only two consecutive β-(1→4) linkages in a polysaccharide could not bind and be hydrolyzed. The avocado enzyme does resemble *Streptomyces* cellulase in that only (1→4)-β- linkages adjacent to another (1→4)-β- were hydrolyzed but would appear to require a longer sequence of (1→4)-β- glucosyl linkages for binding. Such a binding-hydrolytic site requirement would produce a range of oligosaccharides from the *Avena* β-D-glucan molecule (Fig. 6). This may also account for the long incubation time required to reach the extensive hydrolysis that was observed.

Hydrolytic activity against isolated cell walls—In order to assess the effectiveness of cellulase in degrading native cell walls the purified enzyme was incubated with isolated cell walls from avocado fruits. Hayashi et al. (1984) demonstrated that pea endo-1,4-β-glucanase could effectively degrade pea xyloglucan though at a much slower rate than cellodextrins or cellulose derivatives. Cell walls were also treated with a commercial cellulase preparation (Worthington) to determine the extent to which glucose could be released from these cell wall matrices. Table 3 summarizes the results of these experiments. The most effective preparation in degrading the different types of isolated cell walls was the commercial cellulase.

The avocado cellulase resulted in a significant release of carbohydrate from "green" avocado cell walls but a limited release from "ripe" avocado walls. The neutral sugar composition of the released carbohydrate by the purified avocado cellulase (Table 4) indicates however that glucose was a minor component. The major sugars found in the solubilized fraction were arabinose and

Table 3 Total carbohydrate released from isolated cell walls by selected enzyme treatments

Cell wall material	Total carbohydrate released (μg/mg cell wall)		
	Control	Avocado cellulase	Commercial cellulase
Avocado "green"[a]	39.3	167.5	378.4
Avocado "ripe"[b]	65.7	68.0	421.9

[a] "Green" refers to unripe-hard fruits before cellulase activity is detected.

[b] "Ripe" refers to soft fruits at or close to the peak of cellulase activity.

Avocado cellulase is represented by the purified enzyme. The commercial cellulase is from Worthington. Wall preparations were incubated with the appropriate enzyme for 72 h.

Table 4 Neutral sugar composition of carbohydrate released upon treatment of isolated avocado cell walls with a purified avocado cellulase (Enzyme) and by a commercial cellulase (C. Cellulase)

Sugar	Neutral sugar composition[a]					
	Avocado "Green"			Avocado "Ripe"		
	Control[b]	Enzyme[c]	C. Cellulase	Control	Enzyme	C. Cellulase
Rha	—	0.032	0.010	0.036	0.045	0.007
Fuc	—	—	—	—	—	—
Rib	—	—	—	—	—	—
Ara	0.189	0.466	0.211	0.431	0.472	0.138
Xyl	0.077	0.013	0.098	0.164	0.138	0.176
Man	0.109	0.006	0.044	0.072	0.057	0.046
Gal	0.366	0.418	0.225	0.109	0.104	0.062
Glc	0.259	0.038	0.393	0.186	0.173	0.554
?	—	0.017	0.004	—	0.011	0.002

[a] The neutral sugar composition is represented as a ratio of the total mole fraction of neutral sugars hydrolized by 2 M TFA.

[b] Boiled cell walls incubated without enzyme added.

[c] Purified avocado cellulase.

galactose. Although this would suggest enzyme contamination, no such activity was found with the soluble substrates after a 24 h incubation with the purified cellulase. Gel filtration of the solublized sugars on a Fractogel column revealed that most of the carbohydrate eluted in a region corresponding to a molecular weight greater than 10,000. This may indicate that the major released carbohydrate was not due to direct enzymic activity against polysaccharides containing arabinose and galactose. The cell walls from both "ripe" and "green" avocado fruits were degraded to a greater extent by the commercial cellulase (Table 3) and neutral sugar analysis indicated that glucose constituted a greater porportion of the released carbohydrate as compared to the avocado enzyme (Table 4).

The results presented here indicate that the cellulase from avocado does not cause appreciable solubilization of (1→4)-β-D-glucan components within the cell wall matrix. A cellulase complex similar to that proposed by Sobotka and Stelzig (1974) for ripening tomato cannot be ruled out. However, experiments with crude extract which should contain such a complex did not result in an increased solubilization of glucose from isolated avocado cell walls (unpublished results).

Ultrastructural studies of ripening avocado fruits (Platt-Aloia 1980) indicate a loss of cellulose fibrillar orientation during the initial stages which becomes more pronounced as ripening continues. Platt-Aloia et al. (1980) found a complete loss of cell wall material in very soft post-climacteric avocado fruit and suggested that the avocado cellulase was responsible for this ultrastructural observation. The results reported here would suggest that the extractable avocado cellulase cannot account for a complete loss of wall cellulosic material.

Alternatively the role of the cellulase in avocado may be the hydrolysis of specific regions of xyloglucans or cellulose fibrils which could account for changes in cellulose fibrillar orientation. This may also lead to a disruption of hydrogen bonding to other matrix polysaccharides. Although there would appear to be no appreciable release of xyloglucan fragments from avocado cell walls by the avocado enzyme (Table 4), limited hydrolysis may be sufficient to disrupt the cell wall matrix. With limited hydrolysis the xyloglucan may retain sufficient integrity so that products remain associated with cellulose and are not released into the reaction medium. When avocado cell walls isolated from different stages of ripening were extracted with 1 M KOH, there

was an enrichment in glucose and xylose as a fruit softening increased (unpublished results). This observation supports the hypothesis that limited hydrolysis of xyloglucan results in a greater proportion being extracted by 1 M KOH. Limited cleavage of (1→4)-β- glucosidic bonds may not result in a release of detectable glucose but could lead to increased disorientation of the cellulose fibrillar network. Loosening of this matrix may be important to the rapid solubilization of other wall polysaccharides. Such a mechanism may explain why cellulase activity is observed to increase prior to an increase in polygalacturonase activity during the ripening process (Awad and Young 1979). Greater accessibility to polygalacturonans in the wall matrix may account for the rapid changes in fruit firmness in a relatively short period of time.

The cellulase from avocado fruits would be classified as a endo-(1→4)-β-D-glucanase. Hydrolytic activity would appear to be limited to cellodextrins or accessible regions on (1→4)-β-D-glucans of four or more glucosyl residues. This enzyme does not appear to effectively solubilize crystalline cellulose such as Avicel or cellulosic polymers contained within native cell walls. The role of this cellulase in avocado fruit ripening may be one of disrupting and loosening the cell wall matrix rather than one of rapid solubilization.

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